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Characterization of the myosin light chain kinase from smooth muscle as an actin-binding protein that assembles actin filaments in vitro

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Abstract

In addition to its kinase activity, myosin light chain kinase has an actin-binding activity, which results in bundling of actin filaments [Hayakawa et al., *Biochem. Biophys. Res. Commun.* 199, 786–791, 1994]. There are two actin-binding sites on the kinase: calcium- and calmodulin-sensitive and insensitive sites [Ye et al., *J. Biol. Chem.* 272, 32182–32189, 1997]. The calcium/calmodulin-sensitive, actin-binding site is located at Asp²–Pro⁴¹ and the insensitive site is at Ser¹³⁸–Met²¹³. The cyanogen bromide fragment, consisting of Asp²–Met²¹³, is furnished with both sites and is the actin-binding core of myosin light chain kinase. Cross-linking between the two sites assembles actin filaments into bundles. Breaking of actin-binding at the calcium/calmodulin-sensitive site by calcium/calmodulin disassembles the bundles. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

Myosin light chain kinase (MLCK) is an enzyme that phosphorylates the light chain of smooth muscle myosin in the presence of Ca²⁺ and calmodulin (Ca/CaM), thereby activating the myosin so that it can interact with actin filaments (see [1] for review). It thus follows that MLCK has the ability to bind myosin [2]. In addition, MLCK has an actin-binding activity [2–5] that is abolished by Ca/CaM [2,6,7]. This activity, however, is not related to the kinase activity of MLCK and suggests that MLCK has another role in regulating the actin-myosin interaction.

A novel role of MLCK that has previously described is that it binds to actin filaments and inhibits the actin-myosin interaction [8–10]. The inhibition is relieved by Ca/CaM. This actin-linked regulation is similar to that of caldesmon and calponin [11–14], see also [15–17] for reviews. Recently, we have published that MLCK has Ca/CaM-sensitive and insensitive actin-binding sites at its N-terminal and middle portions, respectively [18]. The Ca/CaM-sensitive site corresponds to the site reported by Kanoh et al. [19], Gallagher and Stull [20] and Lin et al. [7].

To date, many actin-binding proteins have been proposed, most of which also modulate the function of actin filaments (see [21] for review). We have demonstrated morphologically and biochemically that MLCK assembles actin filaments into bundles [6]. In this study, we investigate the sites in MLCK

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that cross-link actin filaments and are responsible for its bundling activity.

2. Materials and methods

2.1. Preparation of proteins

All procedures were carried out at 0–4°C. The purity of proteins was routinely monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below) so that they were >95% pure. The purified proteins, except actin (see below), were divided into aliquots and stored at –80°C until they were used. All columns (see below) were incorporated into a high performance liquid chromatography system (model L-6200, Hitachi, Tokyo, Japan). MLCK was prepared by the method of Adelstein and Klee [22] with a slight modification [6]. In short, MLCK was extracted from smooth muscle of chicken gizzard and was subjected to ammonium sulfate fractionation. After fractionation, MLCK was purified using column chromatography in a DEAE-Toyopearl 650M column and subsequently in SP-Toyopearl 650M and AF-blue Toyopearl 650 ML columns (Tosoh, Tokyo, Japan). This MLCK was used in all experiments.

After passing through a DEAE-Toyopearl chromatography column, the MLCK preparation was left on ice overnight. In addition to the parent 130 kDa MLCK band, SDS-PAGE detected a 110 kDa band presumably produced by the endogenous protease(s). This 110 kDa fragment was co-purified with MLCK by subsequent SP-Toyopearl and AF-blue-Toyopearl chromatography. The mixture was used for the actin-binding assay (see below).

MLCK fragments were obtained by the chemical cleavage of MLCK with cyanogen bromide (CNBr) at a Met site or with 2-nitro-5-thiocyanatobenzoic acid (NTCB) at a Cys site. MLCK was cleaved by CNBr using the method of Lucas et al. [23]. Cleavage by NTCB used the method described for caldesmon by Sutherland and Walsh [24]: 6 mg MLCK was incubated at 25°C in 2 mM NTCB, 0.1 mM EGTA and 200 mM Tris-HCl (pH 9.0). After incubation for 12 h, the reaction was terminated by adding 2-mercaptoethanol (2-ME) to a final concentration of 14 mM. The actin-binding fragments, as monitored by

the high-speed centrifugation assay (see below), were 30 kDa for CNBr cleavage and 17 kDa for NTCB cleavage in SDS-PAGE (see below for the actual molecular masses of these fragments). These actin-binding fragments were purified by modifying the method of Kanoh et al. [19] as follows. MLCK subjected to cleavage by CNBr or NTCB was dialysed against 30 mM sodium acetate (pH 3.1), 0.1 mM EGTA and 14 mM 2-ME. After dialysis, the precipitate was removed by centrifugation and the supernatant was applied to a SP-Toyopearl 650M column that was equilibrated in the same buffer. The actin-binding fragments were then eluted by a linear NaCl gradient of 0–0.5 M. The peak samples containing the fragments were pooled and dialysed against 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA and 14 mM 2-ME and were then applied to a column of DEAE-Toyopearl 650M that was equilibrated in the same buffer. The fragments were again eluted by a linear NaCl gradient of 0–0.5 M, concentrated by ultrafiltration with Centricon-10 (Amicon, Beverly, MA, USA) and applied to a Superose 12 gel filtration column (Pharmacia, Upsala, Sweden). After checking the purity of the CNBr and NTCB fragments eluted from the column by SDS-PAGE (see below), the fragments were used in our experiments.

The positions of the NTCB, CNBr and 110 kDa fragments in the primary structure of MLCK [25] were determined by partial amino acid sequencing from the N-terminal of the fragments using an Applied Biosystems 477A analyser. The NTCB fragment consisted of the Met¹–Lys¹¹⁴ sequence (12.4 kDa, see [17]) and the CNBr fragment of the Asp²–Met²¹³ sequence (22.5 kDa). The intact MLCK consisted of the Met¹–Glu⁹⁷² sequence (107.5 kDa, see [25]). The molecular masses used for calculating the molar concentrations of these fragments were obtained from the amino acid sequences.

The recombinant actin-binding fragments of MLCK were prepared as described previously [17,18]. In short, the pET21 vectors carrying cDNAs for the fragments were transfected to *Escherichia coli* BL 21 (DE 3). The fragments were overexpressed and then purified. The fragments were: NN fragments (35.3 kDa) containing a Ca/CaM-sensitive actin-binding site [18], a NC fragment (44.4 kDa) containing a Ca/CaM-insensitive site [18] and a N

fragment (77.4 kDa) containing both the Ca/CaM-sensitive and insensitive sites [17].

Skeletal muscle actin was purified from an acetone powder of chicken breast muscle [26] and used as actin filaments after polymerization. The concentrations of actin filaments were expressed in terms of monomeric actin. CaM from bovine brain was from Sigma (St Louis, MO, USA). We adopted molecular masses of 41.8 kDa for actin and 16.7 kDa for CaM.

2.2. Low-speed centrifugation assay for the actin-bundling activity of MLCK and its fragments

Actin filaments at a concentration of 12 μM were mixed with specified amounts of MLCK and CaM in 50 mM KCl, 20 mM Tris-HCl (pH 7.5) and 1 mM EGTA or 1 mM Ca^{2+} unless otherwise specified in the figure legends. The mixture was incubated for 1 h at 25°C and centrifuged at $11\,000\times g$ for 15 min at 25°C. The supernatants were separated from the pellets and both fractions were subjected to SDS-PAGE (see below). To quantify the bundling activity, the amounts of actin isolated by SDS-PAGE from the supernatant and the pellet were determined with a microdensitometer [6]. The activity was also monitored by optical and electron microscopy (see below).

Unless otherwise specified, Ca^{2+} was at a high concentration (1 mM) for the low- and high-speed centrifugation assays (see below) to ensure that CaM could fully interact with MLCK and its fragments.

2.3. High-speed centrifugation assay for the actin-binding activity of MLCK and its fragments

Actin filaments at a concentration of 12 μM were mixed with specified amounts of MLCK and CaM in 50 mM KCl, 20 mM Tris-HCl (pH 7.5) and 1 mM EGTA or 1 mM Ca^{2+} unless otherwise specified in the figure legends. The mixture was incubated for 1 h at 25°C and centrifuged at $140\,000\times g$ for 20 min in a Beckman Airfuge at 25°C. The amount of MLCK in both the supernatants and the pellets was determined by SDS-PAGE followed by densitometry as described above and then, the amount of MLCK

bound to actin filaments was calculated [11]. The binding activity of the fragments of MLCK was assayed in the same way.

Because the low-speed centrifugation assay precipitates actin filaments assembled by MLCK, isolated actin filaments, even though they may be bound to other actin filaments, remain in the supernatant. However, the high-speed centrifugation precipitates both the assembled and isolated actin filaments, giving the total amount of MLCK bound to actin filaments.

2.4. Interaction between CaM, MLCK and its fragments

CaM was labelled with dansyl-chloride (Wako Pure Chemical, Tokyo, Japan) by a procedure described previously by Kincaid et al. [27]. Increasing amounts of MLCK or its fragments were added at 25°C in 50 mM KCl, 20 mM Tris-HCl (pH 7.5) and 0.1 mM Ca^{2+} or 0.1 mM EGTA and changes in the intensity of the fluorescence of dansyl-CaM (0.04 μM) were measured using a spectrofluorometer (FP-770, Jasco, Tokyo, Japan). An emission spectrum was obtained with an excitation wavelength of 340 nm. Changes in the intensity at 473 nm were considered to indicate the binding of MLCK or its fragments to CaM.

2.5. Other procedures

Actin filaments that were bundled by MLCK or its fragments as described above were observed using a phase-contrast optical microscope (Zeiss Axioplan, Oberkochen, Germany). They were also observed with a JEM 100C electron microscope (Jeol, Tokyo, Japan) after negative staining with 1% uranyl acetate. Just before staining, samples were diluted 3-fold with the buffer used to dissolve them.

The sedimentation coefficient (*S* value) of a 3.2 μM solution of MLCK was determined by measuring its sedimentation velocity with an analytical ultracentrifuge (model 282, Hitachi, Tokyo, Japan) at 60 000 rpm at 20°C by modifying the procedures described by Adelstein and Klee [23].

SDS-PAGE was carried out using the method of Laemmli [28] with a slight modification [11]. Protein concentrations were determined using the method of

Bradford [29] and/or Lowry et al. [30] with bovine serum albumin as the standard.

3. Results

3.1. Bundling of actin filaments by MLCK

Actin filaments were incubated together with MLCK and CaM to form bundles (Fig. 1a). After incubation, the actin filaments were assayed using low-speed centrifugation to quantify the bundling activity of MLCK. In the absence of MLCK, actin filaments were not precipitated (Fig. 2a, lane 2). However, in the presence of MLCK, most of the actin filaments were recovered in the precipitate together with MLCK (Fig. 2a, lane 4).

Fig. 2b shows the quantitation of the bundling activity of MLCK. In the absence of Ca^{2+} , the numbers of actin filaments bundled by MLCK increased with the increase in the concentration of MLCK (open circles). The maximal percentage of bundled actin filaments was $83.3 \pm 3.8\%$ (mean \pm S.E.M.,

$n=4$). The concentration of MLCK giving half-maximal bundling was $1.5 \pm 0.1 \mu\text{M}$, (mean \pm S.E.M., $n=4$), i.e. 8.3 mol actin per mol MLCK. When CaM was allowed to interact with MLCK in the presence of Ca^{2+} , there was virtually no bundling activity (filled circles).

Fig. 2c examines the stoichiometry of CaM:MLCK required to bundle actin filaments. Under conditions in which MLCK assembles 75% of the actin filaments as bundles, we examined the effect of CaM at various ratios to MLCK in the presence of Ca^{2+} . The percentage of bundled actin filaments decreased with the increase in the CaM/MLCK ratio. The CaM/MLCK ratio that gave half-maximal bundling was 0.73 ± 0.07 (mean \pm S.E.M., $n=3$).

3.2. Is polymeric MLCK required for bundling activity?

MLCK in low-salt solutions has been reported to polymerize at higher concentrations [31]. MLCK at a concentration of $3.2 \mu\text{M}$ was subjected to analytical

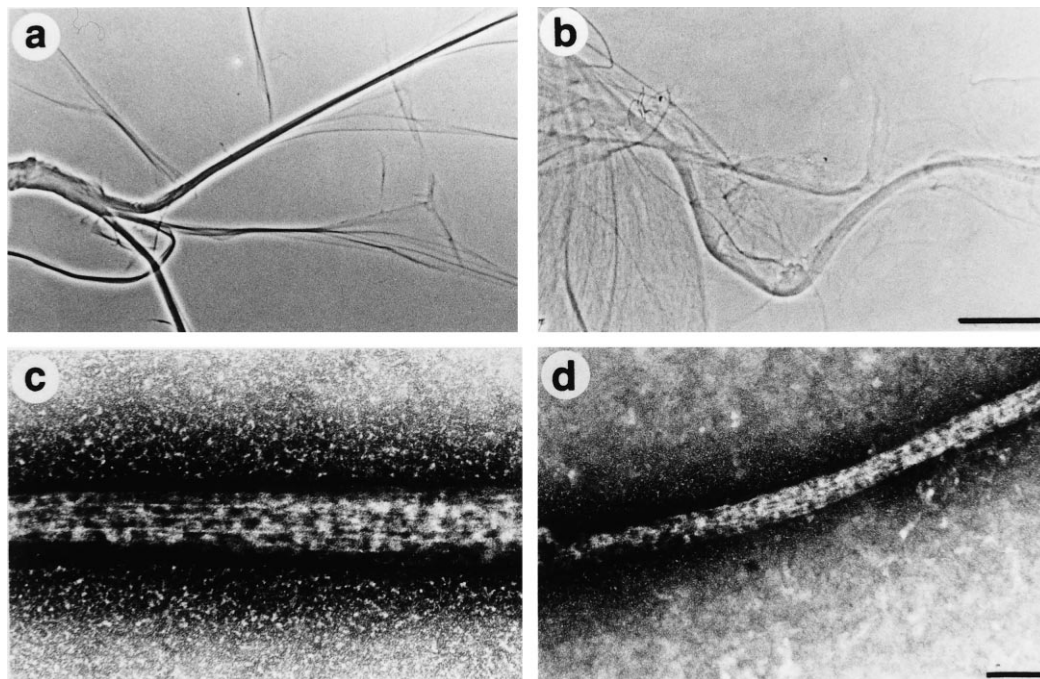


Fig. 1. Bundles of actin filaments as visualized by phase-contrast and electron microscopies. Actin filaments were mixed with $1.5 \mu\text{M}$ MLCK or $8.4 \mu\text{M}$ CNBr fragment and incubated at 25°C for 1 h to form bundles. The bundles were observed with a phase-contrast microscope (a and b) or with an electron microscope (c and d) after negative staining. (a) and (c), MLCK; (b) and (d), CNBr fragment. Bar, $50 \mu\text{m}$ for (a) and (b); $0.1 \mu\text{m}$ for (c) and (d).

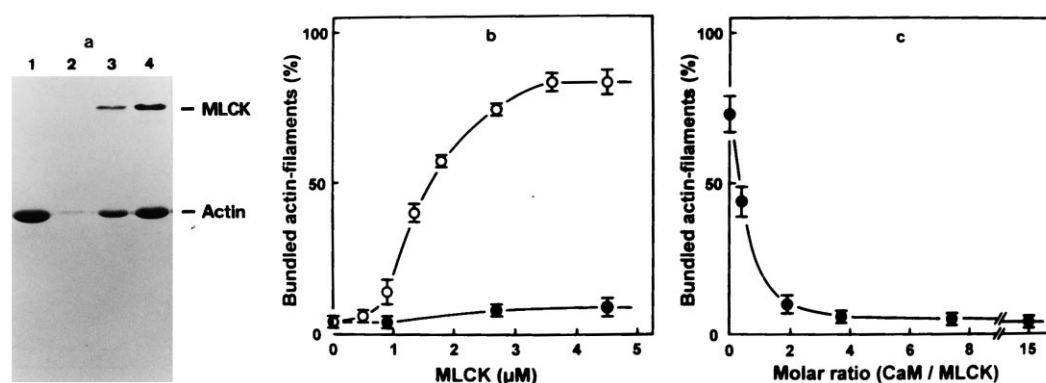


Fig. 2. Bundling of actin filaments by MLCK. Actin filaments were assembled to form bundles as described in the legend to Fig. 1. (a) The bundles of actin filaments were formed in EGTA with or without 2.7 μM MLCK and then precipitated by low-speed centrifugation. The supernatant (lanes 1, 3) and precipitate (lanes 2, 4) were separately subjected to SDS-PAGE. Lanes 1 and 2, without MLCK; lanes 3 and 4, with MLCK. (b) Actin filaments were allowed to form bundles in the various concentrations of MLCK (0–4.5 μM), 20 μM CaM and EGTA or Ca²⁺. The bundles were precipitated by low-speed centrifugation. The percentage of precipitated actin filaments (ordinate) of the total actin was plotted against the concentrations of MLCK (μM) (abscissa). Open circles, in EGTA; filled circles, in Ca²⁺. (c) Actin filaments were assembled by 2.7 μM MLCK, Ca²⁺ and various concentrations of CaM (0–40 μM) and subjected to the low-speed centrifugation assay. The percentage of precipitated actin filaments (ordinate) were plotted against the molar ratio of CaM to MLCK (abscissa). Data are expressed as mean \pm S.E.M. ($n = 3$).

centrifugation in the same buffer used for the bundling activity assay of MLCK. We found that MLCK sedimented with $S_{20,w} = 4.57$, a value that is comparable to the $S_{20,w}$ of the monomeric form (Table 2 in [22]).

MLCK is in its monomeric form under the assay conditions for its bundling activity. This is also indicated by a cross-linking experiment as follows. We incubated MLCK at 2.7 μM in 50 mM KCl, 20 mM Tris-HCl (pH 7.5), 1 mM EGTA and with 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide according to the method described by Sobieszek et al. [31]. The incubated MLCK was then subjected to SDS-PAGE. We found no protein bands that indicated cross-linking between MLCK molecules (data not shown).

Bundling of actin filaments has been attributed to cross-linking between actin-binding sites. Thus, two or more sites are required for the proteins that assemble actin filaments (see [21], for a review). In the case of caldesmon, the multiplicity is attained by forming dimers between sulphhydryl residues in the caldesmon molecule [32]. However, the above experiments indicate that MLCK is present in a monomeric form under the conditions required for actin-bundling activity. Thus, MLCK would be expected

to have two (or more) actin-binding sites, a hypothesis that is supported by the following experiment.

3.3. Binding of MLCK to actin filaments

MLCK was mixed with actin filaments and CaM in the presence or absence of Ca²⁺. The mixture was subjected to high-speed centrifugation to precipitate MLCK bound to actin filaments. A typical example of the relationship between the amount of MLCK bound to actin filaments and the concentration of free MLCK is shown in Fig. 3, inset. Analysis with double reciprocal plots (Fig. 3) indicated that MLCK in the presence of CaM was bound to actin filaments with an apparent association constant (K_a) = $2.2 \times 10^6/\text{M}$ in EGTA and with a $K_a = 5 \times 10^4/\text{M}$ in Ca²⁺ (Table 1). These figures are in agreement with

Table 1
Association constants of MLCK and its fragments

	In EGTA	In Ca/CaM
MLCK	$2.2 \times 10^6/\text{M}$	$5 \times 10^4/\text{M}$
CNBr fragment	$1.1 \times 10^5/\text{M}$	$6 \times 10^4/\text{M}$
NTCB fragment	$2.0 \times 10^5/\text{M}$	N.D.

N.D., not detectable.

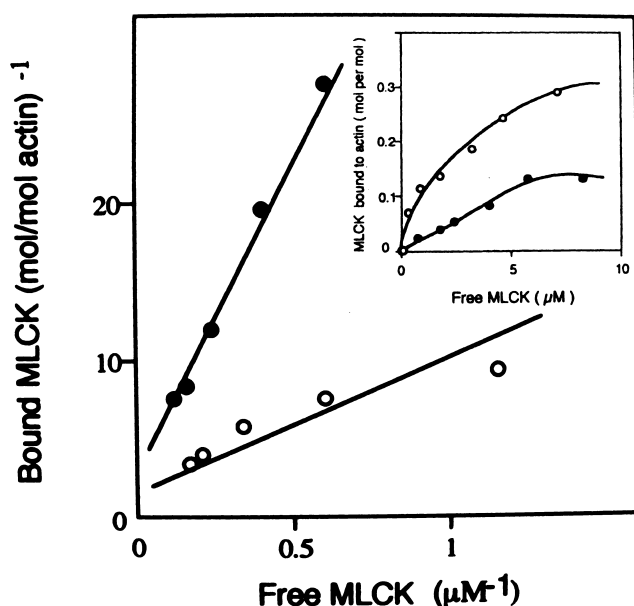


Fig. 3. Binding of MLCK to actin filaments. Actin filaments were mixed with various concentrations of MLCK (0–4.5 μ M), CaM at a 8-fold molar excess over MLCK and EGTA or Ca^{2+} . After incubation, the mixture was subjected to the high-speed centrifugation assay for actin-binding activity. The amounts (mol/mol actin) of MLCK bound to actin filaments (ordinate) were plotted against the concentrations (μ M) of free MLCK (abscissa) as shown in the inset and analyzed by double reciprocal plots. Open circles, with EGTA; filled circles, with Ca^{2+} .

those of Sellers and Pato [2]. However, unlike their report, the inhibition by Ca/CaM was not competitive because the lines of double reciprocal plots in the presence and absence of Ca/CaM did not meet on the ordinate or abscissa. This non-competitive binding property is in accordance with our previous conclusion that MLCK binds to actin filaments in both Ca/CaM-sensitive and insensitive manners [18]. Analysis with Scatchard plots indicated that the binding in EGTA was via high ($K_a = 1.84 \times 10^6/\text{M}$) and low ($K_a = 7.5 \times 10^4/\text{M}$) affinity sites and that the relationship in Ca^{2+} was only via a low affinity site ($K_a = 9.1 \times 10^4/\text{M}$). These data confirm our previous data [18] and give further support for two classes of binding sites.

Because of the monomeric nature of MLCK in the solution used in our experiments, bundling of actin filaments by MLCK can be attributed to the cross-linking of actin filaments by these Ca/CaM-sensitive and insensitive sites. When actin-binding at the Ca/

CaM-sensitive site was abolished by Ca/CaM, bundles of actin filaments should not be assembled (Fig. 2c).

3.4. Binding of the CNBr fragment to actin filaments

To examine which part of the MLCK molecule binds to actin filaments, MLCK was cleaved at its Met residues by treatment with CNBr. The major fragments generated by CNBr treatment were found to be 30, 17 and 15 kDa by SDS-PAGE. The only fragment showing any actin-binding activity was the 30 kDa fragment (data not shown), indicating that no part other than the 30 kDa fragment contains an actin-binding site. We purified the 30 kDa fragment and designated this the CNBr fragment.

The partial amino acid sequence of the CNBr fragment determined from the N-terminal was DFRANLQRQVKPKTLSEEE. According to the complete sequence of MLCK [25], this partial sequence corresponds to Asp²–Glu²⁰. The first and second Met residues from the N-terminal of MLCK were Met¹ and Met²¹³, indicating that the CNBr fragment consists of Asp²–Met²¹³.

Fig. 4a shows the actin-binding activity of the CNBr fragment. The fragment was mixed with actin filaments and CaM and subjected to high-speed centrifugation. In the presence of EGTA, almost all of the CNBr fragment was precipitated with actin filaments (compare lane 1 with lane 2). However, in the presence of Ca^{2+} , the precipitation of the CNBr fragment was much reduced (lanes 3, 4). This actin-binding assay was carried out in the presence of various concentrations of CNBr fragment (Fig. 4b, inset). Double reciprocal plots of the relationship between the amount of CNBr fragment and its free concentration (Fig. 4b) showed that the CNBr fragment in the presence of CaM was bound with a $K_a = 1.1 \times 10^5/\text{M}$ in EGTA and with a $K_a = 6 \times 10^4/\text{M}$ in Ca^{2+} (Table 1). Because of the limitation of our data, we could not definitely conclude that Ca/CaM antagonized in a non-competitive manner. However, the bundling activity, which requires two or more actin-binding sites in the CNBr fragment, favours the idea that the antagonism was not simply competitive.

It must be noted that the CNBr fragment does not contain the well-characterized CaM-binding site

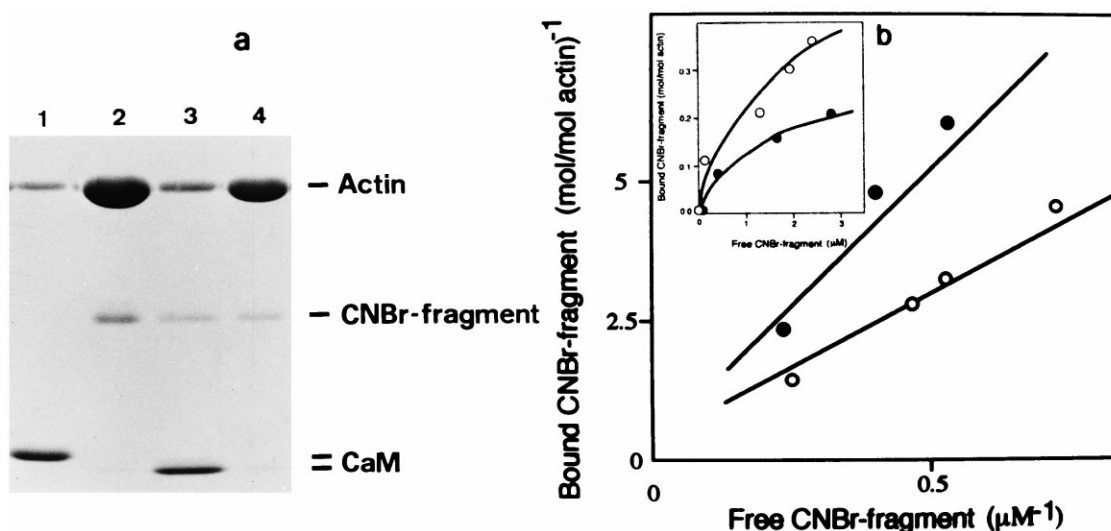


Fig. 4. Binding of the CNBr fragment to actin filaments. We allowed the CNBr fragment of MLCK to bind to actin filaments. (a) Actin filaments were mixed with $8.4 \mu\text{M}$ CNBr fragment, $20 \mu\text{M}$ CaM and EGTA or Ca^{2+} . After incubation, the mixture was subjected to the high-speed centrifugation assay followed by SDS-PAGE. Lanes 1 and 2, in EGTA, and 3 and 4, in Ca^{2+} ; 1 and 3, supernatants after the centrifugation; 2 and 4, precipitates after the centrifugation. (b) Various concentrations (0–10 μM) of the CNBr fragment in EGTA or Ca^{2+} were allowed to bind to actin filaments in the presence of CaM at a 9-fold molar excess over the fragment. The amounts (mol/mol actin) of the fragment bound to actin filaments were plotted against the concentration (μM) of the free fragment as shown in the inset and analyzed by double reciprocal plots. Open circles, EGTA; closed circles, in Ca^{2+} .

(Ala⁷⁹⁶–Ser⁸¹⁵) that regulates the kinase activity of MLCK ([33] for review). Therefore, the effect of Ca/CaM is likely to be through a CaM-binding site in the Ca/CaM-sensitive actin-binding site.

3.5. Bundling of actin filaments by the CNBr fragment

Because the CNBr fragment appears to have two classes of actin-binding sites, we expect that it can assemble actin filaments in a similar way as parent MLCK. To examine this, the CNBr fragment or MLCK was mixed with actin filaments and both mixtures were allowed to assemble. When observed under a phase-contrast microscope, both mixtures showed huge dendritic structures (Fig. 1a and b). Observation with an electron microscope after negative staining revealed that the structures are assemblies of actin filaments (Fig. 1c and d). When Ca/CaM was added to both mixtures, we observed no structure under a phase-contrast microscope, although we observed isolated actin filaments under an electron microscope (data not shown).

We also carried out a quantitative assessment of the bundling activity of the CNBr fragment using the

low-speed centrifugation assay in the presence of various concentrations of the CNBr fragment (Fig. 5a) and confirmed that it assembled actin filaments. However, higher concentrations of the fragment than of parent MLCK were required to induce assembly (compare Fig. 2b with Fig. 5a). As mentioned above, both Ca/CaM-sensitive and insensitive sites cross-link actin filaments to assemble them and the K_a of only the former site is affected upon cleavage by CNBr. Therefore, the need for an increased concentration of the CNBr fragment is attributable to the reduced K_a of its Ca/CaM-sensitive actin-binding site (Table 1).

The effect of Ca/CaM on the assembly was weak compared with parent MLCK. As shown in Fig. 5b, the bundling activity was reduced with increased concentrations of CaM relative to the CNBr fragment in the presence of Ca^{2+} . To prevent the assembly, 12 mol CaM per mol CNBr fragment was required, much higher than that required for parent MLCK (Fig. 2c). We suggest that the affinity for CaM was also reduced upon cleavage by CNBr, which is confirmed by the direct measurement of binding of the CNBr fragment to CaM (see Fig. 7).

Although the affinities of the Ca/CaM-sensitive ac-

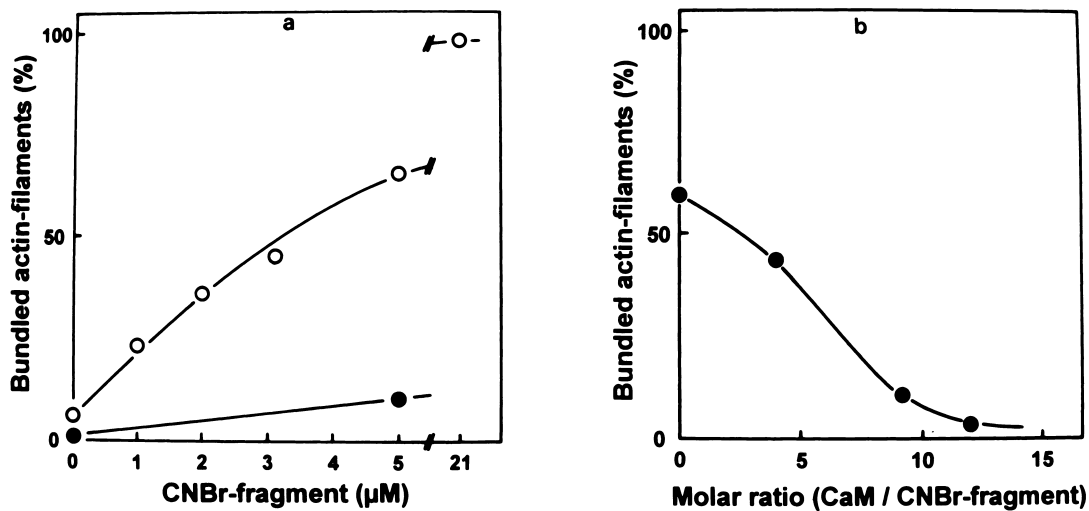


Fig. 5. Bundling of actin filaments by the CNBr fragment. (a) Actin filaments were mixed with various concentrations of the CNBr fragment (0–21 μM) and CaM at a 9-fold molar excess over the CNBr fragment in EGTA or CaCl_2 . The bundling activity of the fragment was examined by the low-speed centrifugation assay. Open circles, in EGTA; filled circles, in Ca^{2+} . (b) The effect of CaM on the actin-bundling activity of 5 μM CNBr fragment was examined in the presence of Ca^{2+} . The amount (%) of the bundled filaments was plotted against the molar ratio of CaM to the fragment. For other details, see the legend to Fig. 2.

tin-binding site and CaM-binding site were reduced, the CNBr fragment contained Ca/CaM-sensitive and insensitive sites and the CaM-binding site. Thus, we see that this fragment is the actin-binding core of MLCK.

3.6. The NTCB fragment consists of only the Ca/CaM-sensitive actin-binding site

Kanoh et al. [19] have obtained an actin-binding fragment from MLCK by cleavage with the 5, 5'-

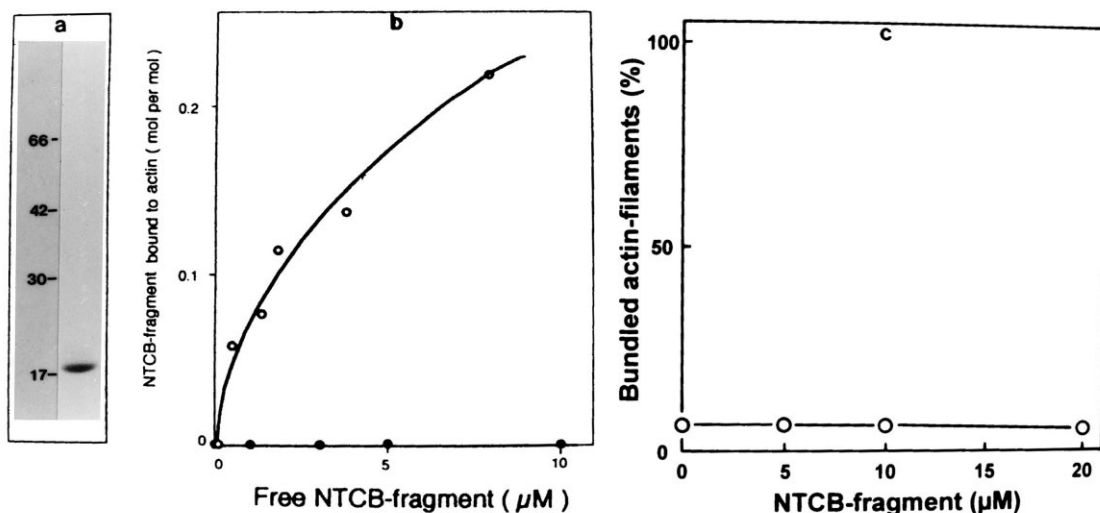


Fig. 6. Binding of the NTCB fragment to actin filaments and bundling of actin filaments by the NTCB fragment. (a) SDS-PAGE of the NTCB fragment. (b) Binding of actin filaments by the NTCB fragment was examined as described in the legend to Fig. 3. The amount (mol/mol actin) of 5 μM NTCB fragment with CaM at a 9-fold molar excess over the NTCB fragment in EGTA (open circles) or Ca^{2+} (filled circles) was plotted against the total concentration of the fragment (μM). (c) Actin filaments were bundled by various concentrations of NTCB fragment (0–20 μM) and CaM at a 9-fold molar excess over the fragment in EGTA as described in the legend to Fig. 2b. N.B. The bundling activity was not detected in this fragment.

dithiobis (2-nitrobenzoic acid)-cyanide complex at Cys residues (see Fig. 6a for SDS-PAGE). We also cleaved MLCK at the same residues by treatment with NTCB (see Section 2). We confirmed their [19] and our [18] reports that the NTCB fragment binds to actin filaments with a single binding constant of $K_a = 2.0 \times 10^5/\text{M}$ (Fig. 6c and Table 1). Our new finding is that the NTCB fragment does not bind to actin filaments in the presence of Ca^{2+} and CaM at a 9-fold molar excess (Fig. 6b). Thus, we conclude that the single actin-binding site of the NTCB fragment is Ca/CaM-sensitive. Its K_a is low compared with the Ca/CaM-sensitive actin-binding site of parent MLCK and is comparable to the K_a of the equivalent site on the CNBr fragment (Fig. 4b). We speculate that the K_a of this site is reduced when it is cleaved from parent MLCK.

We subjected actin filaments mixed with various concentrations of the NTCB fragment to the low-speed centrifugation assay to see whether it assembles actin filaments. As shown in Fig. 6c, we failed to detect any bundling activity by the NTCB fragment. This result confirms our idea that the NTCB fragment contains only a Ca/CaM-sensitive actin-binding site and that both Ca/CaM-sensitive and insensitive sites are required for MLCK to assemble actin filaments.

3.7. Bundling activity as examined by recombinant fragments of MLCK

With the native fragments produced by the chemical cleavage of MLCK, we could not examine whether the Ca/CaM-insensitive actin-binding site alone could bundle actin filaments without assistance from the Ca/CaM-sensitive site. To address such a question, we expressed the recombinant NC fragment, which includes the Ca/CaM-insensitive site [18]. As shown in Fig. 7, the percentages of actin filaments precipitated by the formation of bundles in the presence and absence of the NC fragment were $15.8 \pm 0.2\%$ (mean \pm S.E.M., $n = 3$) and $15.8 \pm 0.6\%$ (mean \pm S.E.M., $n = 3$), respectively, indicating that the Ca/CaM-insensitive site is not able to assemble actin filaments.

Similarly, the NN fragment, analogous to the NTCB fragment, being composed of only the Ca/

CaM-sensitive site, could not assemble actin filaments ($17.1 \pm 0.5\%$, (mean \pm S.E.M., $n = 3$)).

When the N fragment, designed to contain both the NN and NC fragment [17], was mixed with actin filaments, it precipitated them by forming bundles ($76.4 \pm 2.0\%$, (mean \pm S.E.M., $n = 3$)) in a similar way as parent MLCK ($53.5 \pm 1.2\%$, (mean \pm S.E.M., $n = 3$)). The actin-bundling activity of the N fragment confirms the result with the CNBr fragment that cross-linking between Ca/CaM-sensitive and insensitive sites assembles actin filaments into bundles.

These results with the recombinant fragments were in good agreement with those with the native fragments shown in Figs. 2,5,6, except that the N fragment assembled more effectively than parent MLCK. The discrepancy suggests that both Ca/CaM-sensitive and insensitive sites of the N fragment, but not of parent MLCK, are exposed so that they can interact more easily with actin molecules.

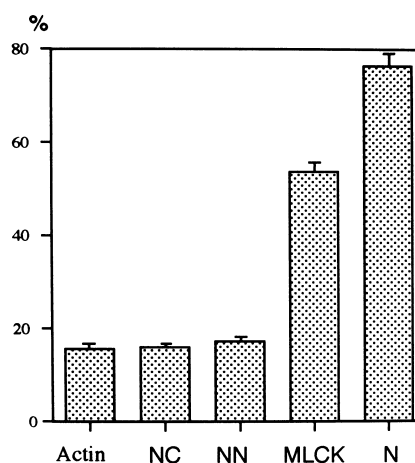


Fig. 7. Bundling of actin filaments by the recombinant fragments of MLCK. Actin filaments ($12 \mu\text{M}$) in 70 mM KCl, 1 mM EGTA and 20 mM Tris-HCl ($\text{pH } 7.5$) were mixed with $3 \mu\text{M}$ of MLCK, the N fragment (N) containing both Ca/CaM-sensitive (2–41 sequence of Fig. 9) and insensitive (138–213 sequence of Fig. 9) actin-binding sites, the NN fragment (NN) containing the Ca/CaM-sensitive actin-binding site or the NC fragment (NC) containing the Ca/CaM-insensitive actin-binding site. The mixtures were subjected to the low-speed centrifugation assay to determine the actin-bundling activity. The percentage of actin filaments precipitated by forming bundles is shown together with the S.E.M. ($n = 3$). Actin refers to the control without MLCK or its fragments.

Table 2

Effects of Ca/CaM on the binding of 130 kDa MLCK and its proteolytic 110 kDa fragment to actin filaments

Proteins	Amount of proteins bound to actin filaments	
	EGTA	Ca ²⁺
MLCK	62.8 ± 12.3%	14.6 ± 3.5%
110 kDa fragment	14.5 ± 9.3%	8.2 ± 4.0%

mean ± S.E.M., *n* = 3.

3.8. Direct detection of the interaction of MLCK and its fragments with CaM

In the previous report [18], CaM-binding activities of parent MLCK and its recombinant fragments were measured by surface plasmon resonance using a CaM-binding cubette with the IAsys Cubette System. In this study, we examined the activities of parent MLCK and its CNBr and NTCB fragments in a different way. The fluorescence emission spectrum of dansyl-CaM changes when Ca²⁺ and/or CaM-binding proteins are present [27]. The fluorescence spectrum of dansyl-CaM in the presence of EGTA exhibited a peak at 518 nm (Fig. 8a, dotted line). Upon addition of CaCl₂ to a final concentration of 1 mM Ca²⁺ (Fig. 8a, broken line), the peak shifted to 507 nm and increased its intensity. Further addition of MLCK (Fig. 8a, continuous line) or its fragments (not shown) increased the intensity and produced a small peak at 473 nm. Therefore, we measured the increase in the intensity at 473 nm to monitor the interaction between CaM and MLCK or its fragments. As shown in Fig. 8b, the intensity increased with an increase in the concentration of the CNBr, NTCB or N fragment in the presence of Ca²⁺. However, the increases for all fragments were so gradual that 6 mol/mol CaM was not sufficient to reach the maximal values. A comparable observation was shown with the NN fragment in Fig. 2a in [16]. The correlation between the results for the fragments confirms that they share a CaM-binding site that regulates the Ca/CaM-sensitive site for actin binding.

Changes in the intensity dye to addition of MLCK are also shown in Fig. 8b. The intensity increased until a molar ratio of between two and three, at which point saturation was reached. MLCK has a CaM-binding site that regulates its kinase activity

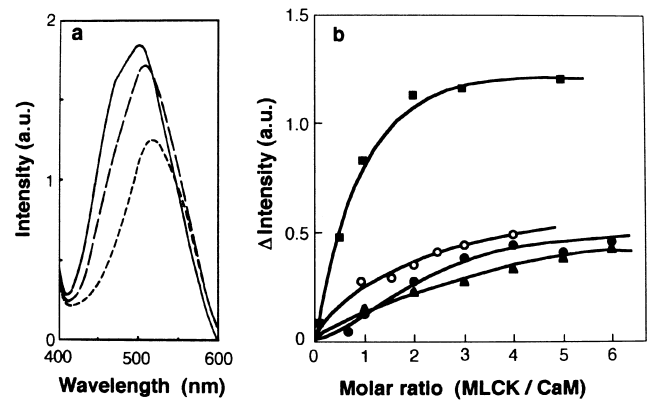


Fig. 8. Interaction of CaM with MLCK, the CNBr fragment and NTCB fragment as measured with dansyl-CaM fluorescence. (a) Emission spectra from dansyl-CaM were obtained as described in Section 2. Dotted line, in the presence of 0.1 mM EGTA; broken line, in the presence of 0.1 mM Ca²⁺; continuous line, in the presence of 0.04 μM MLCK and 0.1 mM Ca²⁺. (b) Fluorescence at 473 nm was recorded by adding MLCK or its fragments to dansyl-CaM cumulatively in the presence of Ca²⁺. The increase in the intensity (ordinate) was plotted against the molar ratios of MLCK and its fragments to CaM (abscissa). Square, MLCK; open circles, N fragment; filled circles, CNBr fragment; triangles, NTCB fragment.

at the site near the kinase domain ([1], for review) in addition to a novel CaM-binding site detected in the CNBr, NTCB and N fragments as described above. Therefore, MLCK interacts with dansyl-CaM at both sites, which explains the larger increase. The feature of the relationship implies that the affinity of MLCK to CaM is the same at both sites. However, this implication remains to be demonstrated more clearly.

3.9. Assignment of actin-binding sites in the MLCK molecule

The high-speed centrifugation assay for the actin-binding activity of the mixture of MLCK and its 110 kDa fragment (Table 2) showed that both bound to actin filaments but that the amount of the bound 110 kDa fragment was lower than that of MLCK. In the presence of Ca/CaM, the binding of MLCK was affected, but the binding of the 110 kDa fragment was not. Therefore, we concluded that the 110 kDa fragment is devoid of the Ca/CaM-sensitive actin-binding site and contains only the Ca/CaM-insensitive site. The partial amino acid sequence from the

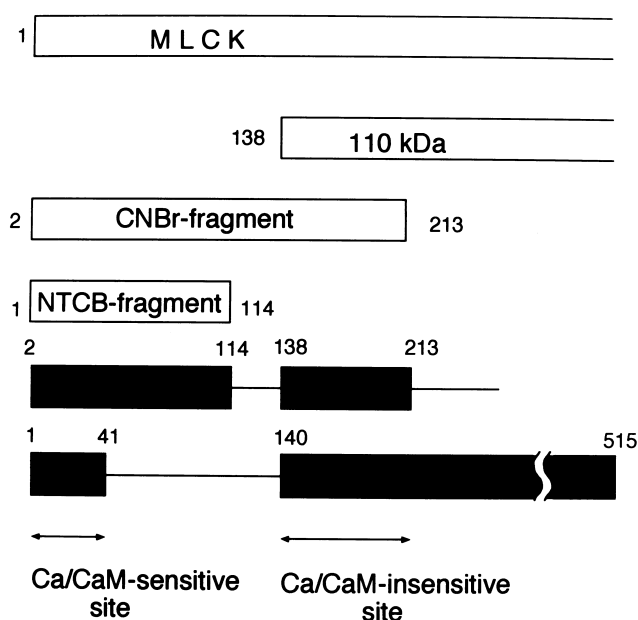


Fig. 9. Schematic diagram of MLCK and its fragments. MLCK, 110 kDa proteolytic fragment, the CNBr-fragment, and the NTCB-fragment are represented by open bars. The upper filled bars are actin-binding sites deduced from the actin-binding activity of these fragments. The lower filled bars are those of previous work with the recombinant fragments (see Fig. 7). The Ca/CaM-sensitive and insensitive, actin-binding sites are assigned by the arrows. The numbers refer to amino acid numbers from the N-terminal of chicken gizzard MLCK (25)

N-terminal of the 110 kDa fragment showed the sequence SKPTPPPSKG, indicating that its N-terminal is Ser¹³⁸. On the other hand, the N-terminal of the 130 kDa band was blocked by acetylation, confirming that it is the parent MLCK molecule.

Fig. 9 compares the structure of parent MLCK with its fragments. Because the actin-binding of MLCK and its fragments was affected by Ca/CaM, their common sequence of Asp²–Lys⁴² must contain the Ca/CaM-sensitive actin-binding site. Studies with the recombinant fragments of MLCK suggest that the Ca/CaM-sensitive site consists of Asp²–Pro⁴¹ [20].

On the other hand, the Ca/CaM-insensitive site would be in the common sequence between the CNBr fragment and the 110 kDa fragment, i.e. Ser¹³⁸–Met²¹³. The recombinant NC fragment, which was expressed from the cDNA of bovine stomach MLCK, is composed of Pro¹⁴⁰–Val⁵¹⁵ [18] of chicken gizzard MLCK [25]. Therefore, the Ca/CaM-insensi-

tive site would be within the sequence of Pro¹⁴⁰–Met²¹³ as shown in Fig. 9.

4. Discussion

We have demonstrated here that MLCK assembles actin filaments into bundles by cross-linking them between its Ca/CaM-sensitive and insensitive actin-binding sites. The CNBr fragment, Asp²–Met²¹³, showed both Ca/CaM-sensitive and insensitive actin binding and assembled actin filaments in a similar way to MLCK. Further, we have shown that this fragment is the sole fragment that showed actin-binding activity when the CNBr digests of MLCK were allowed to bind to actin filaments. Therefore, this fragment is the actin-binding core of MLCK.

The activity of MLCK to assemble actin filaments half-maximally was only 8.3 mol actin/mol MLCK (Fig. 2b), about 2% of an actin filament. Further, the molar ratio of MLCK to actin in smooth muscle cells was between 200 and 300 [19]. Therefore, the bundling activity of MLCK is too low to give it a physiological relevance. However, recent publications report that MLCK in smooth muscle cells is in association with myofibril [7] or myosin light chain phosphatase [34], suggesting the involvement of another protein in increasing the affinity of MLCK for actin. We will find the physiological role of the bundling activity in smooth muscle only when such a protein can be identified.

MLCK shows a Ca/CaM-dependent polymerizing activity, which modifies its kinase activity [31,35–37]. Under conditions that are devoid of Ca/CaM, MLCK is demonstrated to be monomeric by analytical ultracentrifugation. However, the demonstration was insufficient, leaving a possibility that the effect of polymerization might be partially included in the actin bundle formed by parent MLCK shown in Fig. 2b.

MLCK has two distinct CaM-binding sites. The first site is at Ala⁷⁹⁶–Leu⁸¹³ and regulates its kinase activity [25] and the second, at Pro²⁶–Pro⁴¹, regulates its actin-binding activity [18]. To demonstrate that the both sites work in intact MLCK, we synthesized Pro²⁶–Pro⁴¹ and Ser⁷⁸⁷–Ser⁸¹⁵ peptides and allowed them to challenge the regulatory role of Ca/CaM. The former and the latter was effective for actin-

binding and kinase activities, respectively (Fig. 8 in [18]).

To further observe CaM-binding by MLCK, we studied fluorescence from dansyl-CaM as shown in Fig. 8. CaM-binding was saturated at a molar ratio of 2–3, suggesting that each MLCK molecule binds two CaM molecules with a similar affinity. However, the affinity of the CNBr fragment, which contains only the 26–41 site was much lower (Fig. 8b). We speculate that this lower affinity is caused by CNBr cleavage. Similarly, to antagonize the actin-binding (Fig. 3b) and actin-bundling (Fig. 2b) activities of MLCK, 1–2 mol CaM per mol MLCK is sufficient. However, a 12-fold excess of CaM is required to disassemble actin bundles induced by the CNBr fragment (Fig. 5). Thus, reduction in the affinity for CaM at the 26–41 site upon the cleavage was further confirmed. We speculate that the affinity of the 26–41 sequence for CaM is as high as that of the 796–813 sequence and that the former should bind CaM under physiological conditions.

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References

- [1] K.E. Kamm, J.T. Stull, The function of myosin and myosin light chain kinase phosphorylation in smooth muscle, *Annu. Rev. Pharmacol. Toxicol.* 25 (1985) 593–620.
- [2] J.R. Sellers, M.D. Pato, The binding of smooth muscle myosin light chain kinase and phosphatase to actin and myosin, *J. Biol. Chem.* 259 (1984) 7740–7746.
- [3] V. Guerriero Jr., D.R. Rowley, A.R. Means, Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme, *Cell* 27 (1981) 449–458.
- [4] R. Dabrowska, S. Hinkins, M.P. Walsh, D.J. Hartshorne, The binding of smooth muscle myosin light chain kinase to actin, *Biochem. Biophys. Res. Commun.* 107 (1982) 1524–1531.
- [5] K. Yamazaki, K. Itoh, K. Sobue, T. Mori, N. Shibata, Purification of caldesmon and myosin light chain (MLC) kinase from arterial smooth muscle: Comparisons with gizzard caldesmon and MLC kinase, *J. Biochem.* 101 (1987) 1–9.
- [6] K. Hayakawa, T. Okagaki, S. Higashi-Fujime, K. Kohama, Bundling of actin filaments by myosin light chain kinase from smooth muscle, *Biochem. Biophys. Res. Commun.* 199 (1994) 786–791.
- [7] P.-J. Lin, K. Luby-Phelps, J.T. Stull, Binding of myosin light chain kinase to cellular actin-myosin filaments, *J. Biol. Chem.* 272 (1997) 7412–7420.
- [8] K. Kohama, T. Ikagaki, K. Hayakawa, Y. Lin, R. Ishikawa, T. Shimmen, A. Inoue, A novel regulatory effect of myosin light chain kinase from smooth muscle on the ATP-dependent interaction between actin and myosin, *Biochem. Biophys. Res. Commun.* 184 (1992) 1204–1211.
- [9] L.-H. Ye, K. Hayakawa, Y. Lin, T. Okagaki, K. Fujita, K. Kohama, The regulatory role of myosin light chain kinase as an actin-binding protein, *J. Biochem.* 116 (1994) 1377–1382.
- [10] M. Sato, L.-H. Ye, K. Kohama, Myosin light chain kinase from vascular smooth muscle inhibits the ATP-dependent interaction between actin and myosin by binding to actin, *J. Biochem.* 118 (1995) 1–3.
- [11] R. Ishikawa, T. Okagaki, S. Higashi-Fujime, K. Kohama, Stimulation of the interaction between actin and myosin by Physarum caldesmon-like protein and smooth muscle caldesmon, *J. Biol. Chem.* 266 (1991) 21784–21790.
- [12] Y. Lin, L.-H. Ye, R. Ishikawa, K. Fujita, K. Kohama, Stimulatory effect of calponin on myosin ATPase activity, *J. Biochem.* 113 (1993) 643–645.
- [13] Y. Lin, R. Ishikawa, K. Kohama, Role of myosin in the stimulatory effect of caldesmon on the interaction between actin, myosin, and ATP, *J. Biochem.* 114 (1993) 279–283.
- [14] Y. Lin, R. Ishikawa, T. Okagaki, L.-H. Ye, K. Kohama, Stimulation of the ATP-dependent interaction between actin and myosin by a myosin-binding fragment of smooth muscle caldesmon, *Cell Motil. Cytoskeleton* 29 (1994) 250–258.
- [15] T. Okagaki and K. Kohama, Characterization of myosin light chain kinase as an actin-binding protein that regulates the ATP-dependent interaction between actin and myosin of smooth muscle, in: K. Kohama and K. Saida (Eds.), *Smooth Muscle Contraction*, Japan Sci. Soc. Press, Tokyo/S Karger, Basel, Switzerland, 1995, pp. 17–35.
- [16] L.-H. Ye, K. Hayakawa, T. Okagaki and K. Kohama, Actin-binding property of myosin light chain kinase and its role in regulating actin-myosin interaction of smooth muscle, in: T. Nakano and D.J. Hartshorne (Eds.), *Regulation of the Contractile Cycle in Smooth Muscle*, Springer-Verlag, Tokyo, Japan, 1995, pp. 159–173.
- [17] K. Kohama, L.-H. Ye, K. Hayakawa, T. Ikagaki, Myosin light chain kinase: an actin-binding protein that regulates an ATP-dependent interaction with myosin, *Trends Pharmacol. Sci.* 17 (1996) 284–287.
- [18] L.-H. Ye, K. Hayakawa, H. Kishi, M. Imamura, A. Nakamura, T. Okagaki, T. Takagi, A. Iwata, T. Tanaka, K. Kohama, The structure and function of the actin-binding domain of myosin light chain kinase of smooth muscle, *J. Biol. Chem.* 272 (1997) 32182–32189.

- [19] S. Kanoh, M. Ito, E. Niwa, Y. Kawano, D.J. Hartshorne, Actin-binding peptide from smooth muscle myosin light chain kinase, *Biochemistry* 32 (1993) 8902–8907.
- [20] P.J. Gallagher, J.T. Stull, Localization of an actin binding domain in smooth muscle myosin light chain kinase, *Mol. Cell. Biochem.* 173 (1997) 51–57.
- [21] T.D. Pollard, J.A. Cooper, Actin and actin-binding proteins. A critical evaluation of mechanisms and functions, *Annu. Rev. Biochem.* 55 (1986) 987–1035.
- [22] R.S. Adelstein, C.B. Klee, Purification and characterization of smooth muscle myosin light chain kinase, *J. Biol. Chem.* 256 (1981) 7501–7509.
- [23] T.J. Lucas, W.H. Burgess, F.G. Prendergast, W. Lau, D.M. Watterson, Calmodulin binding domains: Characterization of a phosphorylation and calmodulin binding site from myosin light chain kinase, *Biochemistry* 25 (1986) 1458–1464.
- [24] C. Sutherland, M.P. Walsh, Phosphorylation of caldesmon prevents its interaction with smooth muscle myosin, *J. Biol. Chem.* 264 (1989) 578–583.
- [25] N.J. Olson, R.B. Pearson, D.S. Needleman, M.Y. Hurwitz, B.E. Kemp, A.R. Means, Regulatory and structural motifs of chicken gizzard myosin light chain kinase, *Proc. Natl. Acad. Sci. USA* 87 (1990) 2284–2288.
- [26] K. Kohama, Heterogeneity of amino acid incorporation rate in adult skeletal muscle actin, *J. Biochem.* 87 (1980) 997–999.
- [27] R.L. Kincaid, M. Vaughan, J.C. Osborne Jr., V.A. Tkachuk, Ca^{2+} -dependent interaction of 5-dimethylaminonaphthalene-1-sulfonyl calmodulin with cyclic nucleotide phosphodiesterase, calcineurin, and troponin I, *J. Biol. Chem.* 257 (1982) 10638–10643.
- [28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [29] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [30] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [31] A. Sobieszek, A. Strobl, B. Ortner, E.B. Babiychuk, Ca^{2+} -calmodulin-dependent modification of smooth-muscle myosin light-chain kinase leading to its co-operative activation by calmodulin, *Biochem. J.* 295 (1993) 405–411.
- [32] W.P. Lynch, V.M. Riseman, A. Bretsher, Smooth muscle caldesmon is an extended flexible monomeric protein in solution that can readily undergo reversible intra- and intermolecular sulfhydryl cross-linking, *J. Biol. Chem.* 262 (1987) 7429–7437.
- [33] B.G. Allen, M.P. Walsh, The biochemical basis of the regulation of smooth muscle contraction, *Trends Biochem.* 19 (1994) 362–368.
- [34] A. Sobieszek, J. Borkowski, V.S. Babiychuk, Purification and characterization of a smooth muscle myosin light chain kinase-phosphatase complex, *J. Biol. Chem.* 272 (1997) 7034–7041.
- [35] A. Sobieszek, Calmodulin-dependent autophosphorylation of smooth muscle myosin light chain kinase: intermolecular reaction mechanism via dimerization of the kinase and potentiation of the catalytic activity following activation, *Biochemistry* 34 (1995) 11855–11863.
- [36] E.B. Babiychuk, V.S. Babiychuk, A. Sobieszek, Modulation of smooth muscle myosin light chain kinase activity by Ca^{2+} /calmodulin-dependent, oligomeric-type modifications, *Biochemistry* 34 (1995) 6366–6372.
- [37] A.M. Filenko, V.M. Danilova, A. Sobieszek, Smooth muscle myosin light chain kinase, supramolecular organization, modulation of activity, and related conformational changes, *Biophys. J.* 73 (1997) 1593–1606.